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PROCESSING AND PRODUCTS

Evolution of proteolytic indicators during storage of broiler wooden breast meat

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ABSTRACT In the past few yr, an emerging muscle abnormality termed wooden breast (WB) was found to affect broilers' *Pectoralis major* muscles. Although different studies have been performed in order to evaluate the effect of WB on meat quality, there is no evidence concerning its impact on the proteolytic processes taking place during meat aging. Thus, this study aimed at investigating the effect of a 7-day storage of broiler breast fillets on free calcium concentration, calpain activity, and proteolysis. Both the superficial and the deep layers of the *Pectoralis major* muscles were considered. Although similar electrophoretic profiles were observed by comparing the corresponding sampling positions, an evident lack of a high-molecular weight protein band, ascribed to nebulin, was found in the superficial layer of the WB fillets at 10 h postmortem. Compared to normal fillets (NB), both the superficial and the deep layer of WB exhibited a significantly higher amount of free calcium at 168 h postmortem (96 and 88 vs. 20 and 53 μM; $P \leq 0.001$). Casein zymograms evidenced the presence of μ/m-calpain and its autolyzed form migrating as a doublet within the gel. Interestingly, neither the occurrence of WB nor the intra-fillet sampling position exerted any relevant effect on calpain activity. Indeed, a significant reduction ($P \leq 0.05$) in the unautolyzed μ/m-calpain activity coupled with a remarkable increase ($P \leq 0.05$) in the autolyzed form activity was observed during storage. Concurrently, if compared to NB, a significantly larger ($P \leq 0.05$) amount of desmin was detected in both the superficial and the deep layers of the WB samples at 10 h postmortem. Then, a sharp decrease of the intact desmin band coupled with a progressive accumulation of its 39-kDa degradation fragment was observed without any significant difference among groups. In conclusion, the increased hardness that typically affects the WB cases seemed not to be exclusively attributable to differences in the proteolytic processes taking place within the postmortem period.

Key words: broiler, wooden breast, calpain, calcium, desmin

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INTRODUCTION

In the past few yr, the poultry industry has faced an emerging muscle abnormality termed wooden breast (**WB**) whose incidence dramatically rose up, reaching alarming rates. Indeed, in a recent study performed with flocks raised using commercial diets, a remarkable increase in the occurrence of WB condition to 96.1% was observed (Tijare et al., 2016). The WB condition was described by Sihvo et al. (2014) as the appearance of visually hard, out-bulging, and pale areas within the cranial and caudal part of the *Pectoralis major* muscles. The occurrence of WB abnormality was found to be higher in fast-growing and heavier broilers (Petracci et al., 2015). Thus, it is reasonable to hypothesize that pectoral muscles hypertrophy as well as the selection for high growth rate and breast yield might act as direct and/or indirect promoting factors.

A complex aetiology is associated with the occurrence of the WB condition. Previous studies performed in order to characterize the WB abnormality evidenced that, similar to the white-striping defect, the occurrence of WB negatively impacts several aspects of meat quality, including histology, quality traits, and composition. In addition, the profound myodegenerative processes taking place within the WB-affected muscles led to altered water-holding capacity that resulted in overall inferior processing performances of the meat (Sihvo et al., 2014; Mudalal et al., 2015; Soglia et al., 2016a,b; Tijare et al., 2016). Furthermore, as a consequence of their remarkably altered proximate composition (low protein coupled with high moisture, collagen, and fat contents), WB muscles exhibited an impaired nutritional profile (Soglia et al., 2016a,b) and higher protein and lipid oxidation levels (Soglia et al., 2016b). In addition, when associated with the white-striping defect, the WB

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cases exhibited differentially expressed genes related to several functional categories, including muscle development, polysaccharide and glucose metabolism, proteoglycans synthesis, inflammation, and calcium signaling pathway as well (Zambonelli et al., 2016).

Meat tenderizes after slaughter due to the presence of endogenous proteolytic enzymes. The process has been suggested to be multi-catalytic in nature (Kemp and Parr, 2012; Ouali et al., 2013), including the action of calpains, cathepsins, caspases, and the proteasome. There is strong evidence for the involvement of the calpain system in postmortem cleavage of myofibrillar and myofibrillar-associated proteins (Koohmaraie and Geesink, 2006; Huff-Lonergan et al., 2010), and the best-characterized isoforms in mammalian muscle are μ-calpain, m-calpain, and the muscle-specific calpain-3. Birds contain a special variant termed μ/m-calpain with a calcium sensitivity intermediate between those of mammalian μ-calpain and m-calpain (Sorimachi et al., 1995). In chicken, Lee et al. (2007, 2008) described a calpain band in casein zymography with slightly greater motility than that of μ/m-calpain, and this band has been identified as partial autolyzed μ/m-calpain (Zhao et al., 2016). The calpain system in broilers has previously been studied in relation to factors such as animal growth rate (Piórkowska et al., 2015) and early postmortem pH decline rate (Huang et al., 2016). Although different studies have aimed at evaluating the effect exerted by the WB abnormality on meat quality, there is no evidence concerning its impact on the postmortem proteolytic processes. Thus, considering the lack of information concerning proteolytic enzyme activity in WB samples, this study aimed at investigating the effect of a 7-day storage of broiler breast fillets on free calcium concentration, calpain activity, and proteolysis. The superficial layer of the breast fillet was recently described as much harder than the deep layer (Soglia et al., 2017), and, therefore, both the superficial and the deep layers of the *Pectoralis major* muscles were studied in order to understand whether any distinctive traits might be found according to the intra-filet sampling location.

MATERIALS AND METHODS

Sample Collection

A total of 60 boneless and skinless *Pectoralis major* muscles was randomly selected from the same flock of high-breast-yield hybrids (males, Ross 508, 37 to 38 d old, having an average weight of 2.5 kg) in the deboning area of a commercial processing plant (HKScan, Eura, Finland). Thirty WB cases (severely affected muscles exhibiting diffused hardened consistency and macroscopic changes) and 30 normal fillets (**NB**) were selected in 2 batches at 3 h postmortem by 2 experienced people through manual palpation and by visual appearance, kept at 0 to 2°C on ice, and sampled at 10 h postmortem. Only severe WB cases were considered within this study. After trimming off any superficial fat, cartilage, and connective tissue, both NB and WB fillets were packed into a loose polyethylene bag and stored at 5 ± 1°C for 10, 24, 72, 120, and 168 h postmortem. At each time point, 6 NB and 6 WB fillets were sampled and cut in order to separate the surface from the deep layer according to the sampling procedure adopted in our previous study (Soglia et al., 2017). Then, sub-samples were excised from the superficial and deep layers of each sample, coarsely cut (to avoid an excessive fragmentation of the muscle tissue that could influence the analysis) by using a kitchen blender, and used to assess free calcium (from fresh sample), SDS-PAGE, Western blot, and calpain activity (on sample frozen in liquid nitrogen and stored at −80°C). Being the WB samples diffusely affected cases, our sampling protocol led us to analyze both the lesion (surface) and the non-lesion (deep layer) areas of the WB samples and compare them with their corresponding portion within the NB. This sampling protocol was adopted in order to verify whether the WB condition affects the *Pectoralis major* muscles with different degrees of severity that are independent of the sampling position itself.

Free Calcium Concentration

Free calcium concentration was determined on both the superficial and deep layers of 30 NB and 30 WB samples (n = 6 samples/group/storage time) following the procedure described by Pomponio and Ertbjerg (2012) with slight modifications. Briefly, 25 g of chopped breast meat were centrifuged at 18,000 × g for 30 min at 4°C and the supernatant collected. After that, 60 μl of 4 M KCl were added to an aliquot (3 mL) of the supernatant in order to provide a background ionic strength, and the free calcium concentration was measured using a calcium ion selective electrode (**ISE**) equipped with a reference electrode (perfectION™ Combination Calcium Electrode, Mettler Toledo AG, Greifensee, Switzerland), having a measurement range from 0.5 μM to 1.0 M. All measurements were run in duplicates at a constant temperature (20 ± 1°C), and a calibration curve was prepared before performing each run in order to convert the output in mV to a calcium concentration in mM.

Extraction Procedure for SDS-PAGE and Western Blot Analysis

Myofibrils were extracted from both the superficial and the deep layers of 30 NB and 30 WB (n = 6 samples/group/storage time) at different storage times (10, 24, 72, 120, and 168 h postmortem) following the procedure described by Joo et al. (1999) with slight modifications. One g of frozen (−80°C) *Pectoralis major* muscle was homogenized using an IKA Ultra-Turrax T25 homogenizer (Labortechnik, Staufen, Germany) (13,500 rpm for 30 s) in 10 mL of cold rigor buffer

(75 mM KCl, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM EGTA) (pH 7.0). The homogenate was centrifuged at 10,000 × g (4°C for 20 min) and the supernatant containing mainly sarcoplasmic protein discarded. The procedure was repeated twice and the final pellet resuspended in 10 mL of cold rigor buffer. The protein concentration was determined in triplicates by using the RC DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA), and a standard curve was prepared with bovine serum albumin (**BSA**) as a reference.

SDS-PAGE Analysis

In order to avoid any variability in the amount of proteins loaded within each lane, the protein concentration was adjusted to 1.54 μg/μl by adding different amounts of rigor buffer. Then, the samples for SDS-PAGE analysis were prepared according to the procedure described by Liu et al. (2014). In detail, 12.5 μl of NuPAGE® LDS (lithium dodecyl sulfate) Sample Buffer (4 x) and 5 μl of NuPAGE® Sample Reducing Agent (10 x) supplied by Invitrogen (Carlsbad, CA) were added to 32.5 μl of myofibrillar protein samples, and the mixture was heated at 100°C for 3 min in a dry bath heater. Eight μl of sample (corresponding to 8 μg of protein) and 4 μl of Novex® Sharp Pre-Stained Protein Standard were loaded onto the wells of a NuPAGE® Novex® 7% (35 kDa-180 kDa) Tris-Acetate gel (Invitrogen, Carlsbad, CA). Gels were assembled in an XCell SureLock® Mini-Cell electrophoresis chamber and run for 80 min at 150 V by using Tris-Acetate Buffer (Invitrogen, Carlsbad, CA). After staining with Coomassie Brilliant Blue R-250 and acquiring images by a digital camera, myofibrillar protein bands were identified using a Protein Simple AlphaImager® HP System, San Jose, CA.

Western Blot Against Desmin

After being separated through the SDS-PAGE gel, the myofibrillar proteins were electrically transferred to an ImmobilonTM-FL Transfer Membrane (Millipore, Bedford, MA) by a semi-dry transfer system. The blotting process was run at 30 V for 1 h at 4°C. After blocking in 20 mL TBS solution (50 mM Tris, 150 mM NaCl; pH 7.5) and 1 g (50 g/L) of skimmed milk powder at room temperature for 1 h, the membrane was washed with TBS-T (50 mM Tris, 150 mM NaCl, 0.5 g/L Tween-20; pH 7.5) for 10 minutes. Then, in order to detect desmin, membranes were subsequently incubated at room temperature for 1 h with 4 μl of a primary antibody, a mouse antidesmin monoclonal antibody (DE-R-11; Santa Cruz, CA), diluted at 1:5000 in TBS-T and 0.4 g (20 g/L) skimmed milk powder. After that, membranes were subsequently incubated 1 h in the dark with 1 μl of a secondary antibody, a donkey anti-mouse IgG (H+L) antibody (IRDye® 800 CW; Santa Cruz, CA) diluted in 15 mL TBS-T, containing 20 g/L of skimmed milk powder and 0.01 g/L SDS. After washing twice with TBS-T and once with TBS, the Western blot membranes were scanned with a LI-COR® Odyssey Infrared Imaging System-CLx (LI-COR Cop, Lincoln, NE) at 800 nm, and the relative intensities of each band were calculated, considering as 100% the intensity of the desmin band detected in NB at 10 h postmortem.

Casein Zymography

Calpain activity was determined according to the procedure described by Pomponio and Ertbjerg (2012) (n = 6 samples/group/storage time). Each sample was run in duplicate using 12.5% casein precast gels (Bio-Rad Laboratories, Hercules, CA). Briefly, 1 volume (15 μl) of sample buffer (300 mM Tris, 40% glycerol, 0.02% bromophenol blue, 100 mM DTT; pH 6.8) was mixed with 3 volumes (45 μl) of sample. After loading 15 μl of sample, the separation was carried out at 80 V for 3 h at 4°C in 25 mM Tris, 192 mM glycine, and 1 mM EDTA (pH 8.3) running buffer. The gels were subsequently incubated in 100 mL of incubation buffer (50 mM Tris, 10 mM monothioglycerol, 4 mM $CaCl_2$; pH 7.5) and shaken at room temperature for 1 h (3 changes of buffer). Gels were then washed overnight (16 h) in 20 mM Tris and 10 mM EDTA (pH 7.0) in order to stop the calpain activity. After staining with Coomassie Brilliant Blue G-250 and destaining with water (overnight), the calpain activity was visualized as clear bands in a blue gel. The result for each band was expressed as relative intensity (%), considering the band assigned to native μ-m calpain in NB samples at 10 h postmortem as 100%.

Statistical Analysis

The findings were statistically evaluated with the 2-way ANOVA option of the GLM procedure present in SAS software. The main effects of meat abnormality coupled with the sampling position (NB superficial, NB deep, WB superficial, WB deep), the storage time, and their interactions were evaluated and means separated using the Tukey's HSD test (multiple-range test) of the GLM procedure.

RESULTS AND DISCUSSION

The postmortem proteolytic processes taking place within the *Pectoralis major* muscles were studied in order to improve the current knowledge concerning the effect exerted by the WB condition on proteolysis as well as proteolytic enzyme activity in breast meat.

Figure 1 shows the electrophoretic patterns of the myofibrillar proteins extracted from chicken *Pectoralis major* muscles at different postmortem times (10, 24, 72, 120, and 168 h after slaughter). Several myofibrillar protein bands, including nebulin (around 700 kDa), MHC (200 kDa), α-actinin (103 kDa), actin (43 kDa), and tropomyosin (36 kDa), were identified

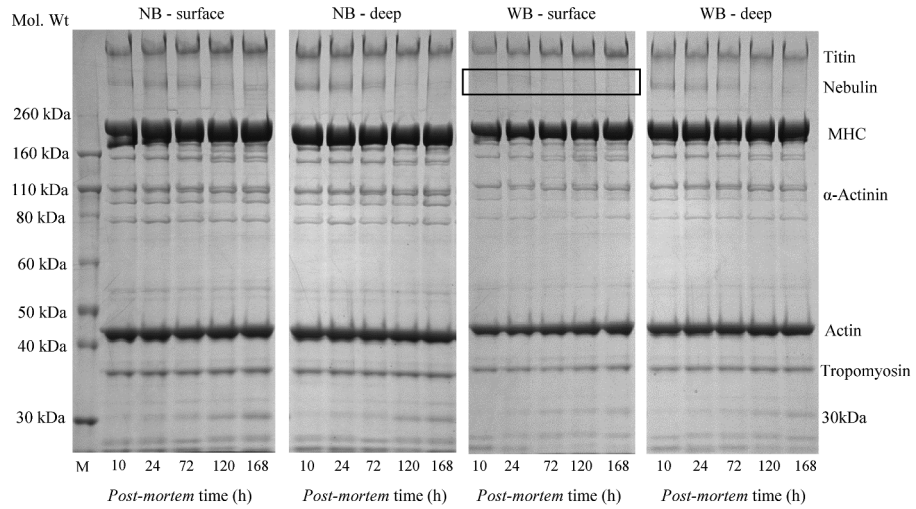

Figure 1. 7% Tris-Acetate gel electrophoresis of myofibrillar proteins extracted from both the superficial and the deep layers of normal (NB) and wooden breast (WB) chicken *Pectoralis major* muscles at different postmortem times (10, 24, 72, 120, and 168 h). M = Molecular weight marker.

Table 1. Postmortem changes in free calcium concentration (μM) measured through an ion selective electrode on both the superficial and the deep layers of normal (NB) and wooden breast (WB) chicken *Pectoralis major* muscles at different times postmortem (10, 24, 72, 120, and 168 h). Mean values ± standard deviation.

		Postmortem time (h)				
		10	24	72	120	168
Group	NB—surface	65 ± 27^{a}	20 ± 5^{b}	25 ± 8^{b}	24 ± 10^{b}	20 ± 7^{b,y}
	NB—deep	55 ± 24	18 ± 6	19 ± 9	27 ± 8	53 ± 13^{x,y}
	WB—surface	57 ± 9^{a,b}	51 ± 13^{b}	45 ± 19^{b}	19 ± 5^{b}	96 ± 43^{a,x}
	WB—deep	47 ± 20^{b}	18 ± 4^{b}	17 ± 3^{b}	53 ± 27^{a,b}	88 ± 29^{a,x,y}

^{a,b}Mean values within the same row followed by different superscript letters significantly differ among the storage times ($P \leq 0.05$).

^{x,y}Mean values within the same column followed by different superscript letters significantly differ among groups ($P \leq 0.05$).

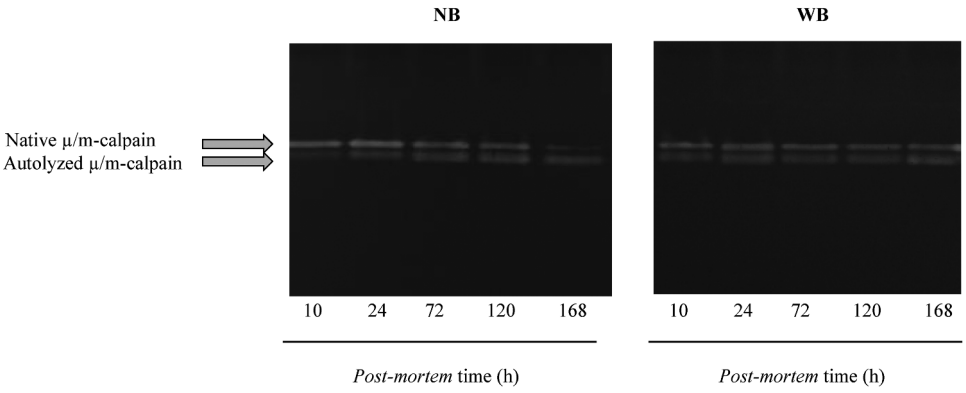

Figure 2. Zymograms showing activity of native (upper left side arrow) and autolyzed (lower left side arrow) μ/m-calpain assessed on the superficial layers of both normal (NB) and wooden breast (WB) chicken *Pectoralis major* muscles at different times postmortem (10, 24, 72, 120, and 168 h).

according to their molecular weight and migration patterns (Lowey et al., 1969; Sakakibara and Yagi 1970; Samejima and Wolfe, 1976; Schiaffino and Reggiani, 1996; Tomaszewska-Gras et al., 2011). An overall increase in the number of detectable bands was observed after aging both in the NB and the WB samples as a result of the proteolytic processes affecting the myofibrillar protein fraction during storage. In agreement with previous studies performed on avian muscles, a 30-kDa degradation product, presumably partly resulting from the hydrolysis of troponin T (Lee et al., 2008), appeared and accumulated during aging of both the NB and the WB fillets. Accumulation of the 30-kDa peptide occurred at a similar rate in NB and WB

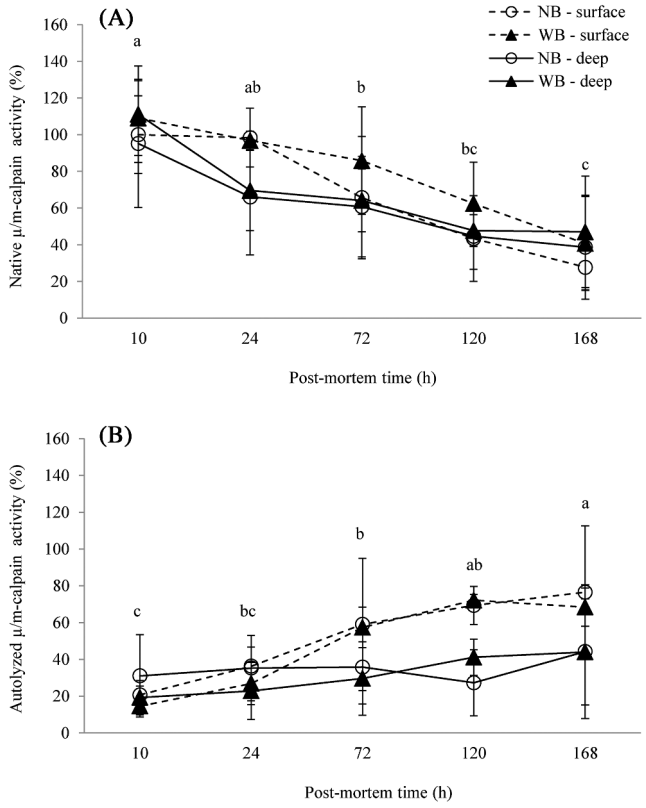

Figure 3. Effect of different postmortem storage times (10, 24, 72, 120, and 168 h) on native (A) and autolyzed μ/m-calpain (B) activity (expressed as %) assessed on both the superficial (dotted line) and the deep (solid line) layers of normal (NB) (round-shaped, open) and wooden breast (WB) (triangle-shaped, solid black) *Pectoralis major* muscles with casein zymography. Error bars indicate standard deviations. [a, b, c]Mean values having different superscript letters significantly differ among the storage times ($P \leq 0.001$).

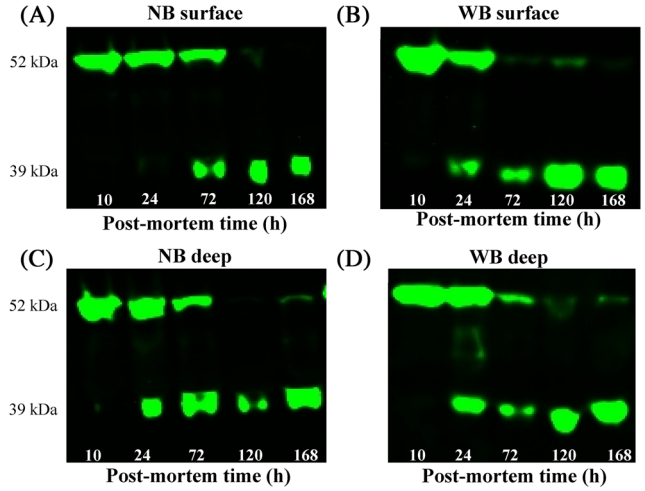

Figure 4. Representative Western blot showing the postmortem changes in desmin from chicken *Pectoralis major* muscles extracted from both the superficial and the deep layers of normal (A, C) and WB affected muscles (B, D).

fillets. In contrast, previous studies reported myofibrillar protein degradation in dystrophic chickens to occur 2-fold faster than within their unaffected counterpart (Hillgartner et al., 1981; Ashmore, Summers and Lee, 1986). On the other hand, although similar electrophoretic profiles were observed comparing the same sampling position (superficial vs. superficial and deep vs. deep layers) for NB and WB samples, an evident lack of a high-molecular weight band, ascribed to nebulin, was found in the superficial layer of the WB fillets at 10 h postmortem. This suggests that nebulin is lacking in the area of the WB-affected muscles characterized by increased hardness—the superficial layer (Soglia et al. 2017). Nebulin is involved in regulation of the structure of thin (actin) filaments and interacts with the Z-disc architecture (Pappas et al. 2008). We speculate that lack of nebulin is related to the structural alterations observed in the muscle fibers of WB observed by Sihvo et al. (2014) and Soglia et al. (2017). Alternatively, the lack of nebulin might be explained by rapid postmortem degradation; however, this explanation is not supported by the presented data on calpain, the appearance of the 30-kDa fragment, desmin degradation pattern, or free calcium early postmortem. Proteolysis of nebulin during aging was previously found to occur in chicken muscles starting from 3 h postmortem by both SDS-PAGE analysis (Paxhia and Parrish, 1988; Chou et al., 1994) and Western blot (Tomaszewska-Gras et al. 2011). Nevertheless, conversely to what was previously observed by Tatsumi and Takahashi (1992), the amount of free calcium measured within this study through an ISE might not contribute in explaining the increased degradation rate of (or lack of) nebulin observed within the superficial layer of the WB samples. As shown in Table 1, no significant differences were found between the 2 experimental groups in the total amount of free calcium assessed at each storage time, with the only exception being the amount measured at 168 h postmortem in which the WB samples (both the superficial and the deep layers) exhibited a significantly higher free calcium content in comparison with the NB (96 and 88 vs. 20 and 53 μM; $P \leq 0.001$). In addition, although the amount of free calcium measured in NB was slightly affected by aging, a remarkable increase was observed within the WB cases (both in the superficial and the deep layers) at 168 h postmortem. The findings of the present study thus support previous studies performed on normal chicken muscles, showing that the free calcium reached its ultimate concentration within the first 24 h postmortem (Nakamura, 1973; Ji and Takahashi, 2006).

In contrast with a previous study performed by Hay et al. (1973) on normal chicken breast muscles, the calcium sequestering properties of the sarcoplasmic reticulum of the WB cases might be impaired during postmortem aging. As a result, a gradual leakage of calcium ions into the sarcoplasm during aging might contribute to explain the presence of significant differences between NB and WB cases at 168 h postmortem. Similarly, an increased level of free calcium was previously observed in *mdx* mice, which are models for studying the Duchenne muscular dystrophy (Turner et al., 1988). Also, the profound changes occurring within the

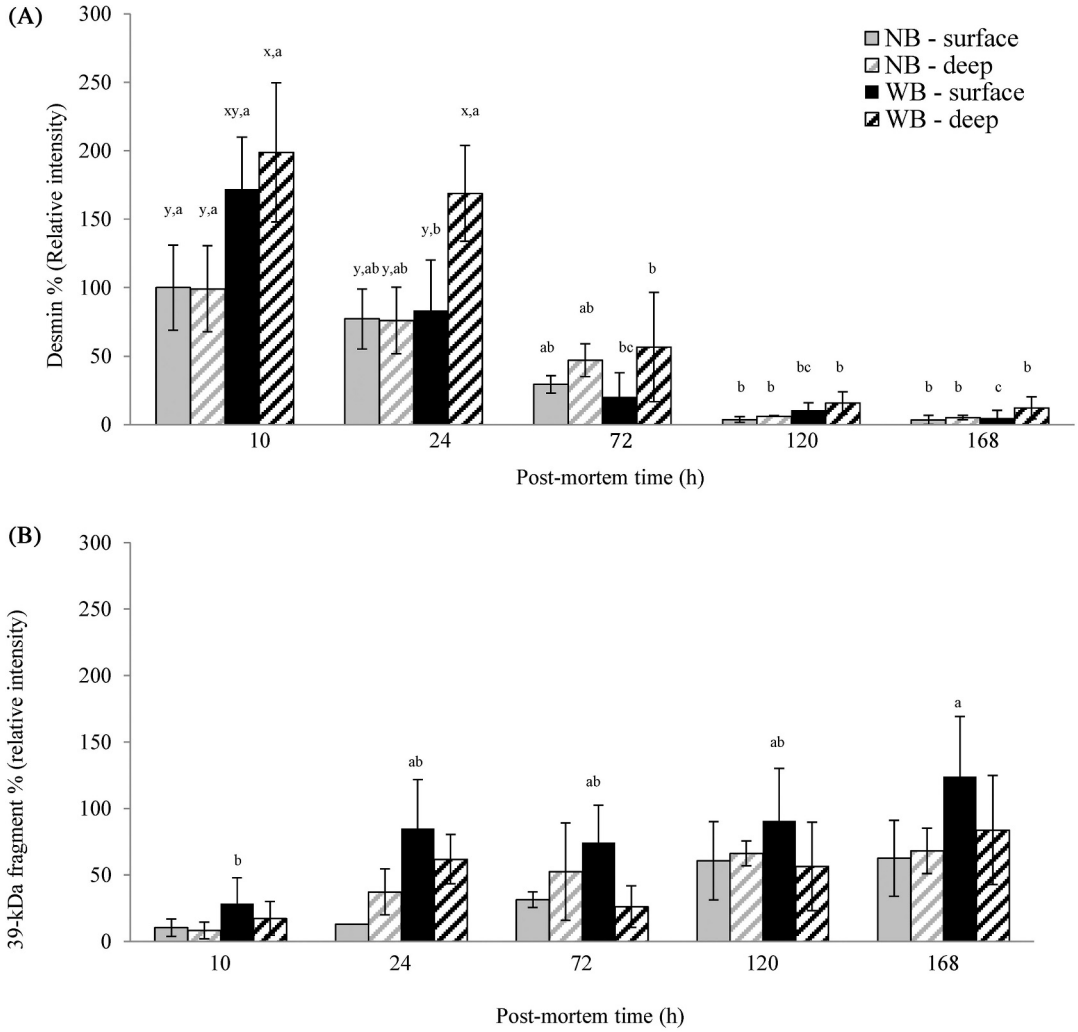

Figure 5. Effect of different postmortem storage times (10, 24, 72, 120, and 168 h) on intact desmin band (A) and its 39-kDa degradation fragment (B) (expressed as %, considering as 100% the intensity of the band assigned to intact desmin in NB samples at 10 h postmortem) assessed on both the superficial (solid bars) and the deep (diagonal striped) layers of normal (NB) (light gray) and wooden breast (WB) (black) *Pectoralis major* muscles by Western blot. Error bars indicate standard deviations. [a, b, c]Mean values having different superscripts significantly differ among the postmortem storage times ($P \leq 0.001$). [x, y]Mean values having different superscripts significantly differ among the experimental groups ($P \leq 0.001$).

postmortem period involving the release of calcium ions as a result of the higher proteolytic degradation of calcium sequestering interfilament proteins (such as titin and nebulin) (Bond and Warner, 2007) might contribute in explaining the higher amount of free calcium observed in WB samples at 168 h postmortem.

In the present study, casein zymography was perfomed on both the superficial and the deep layers of NB and WB *Pectoralis major* muscles in order to assess the activity of both the native and the autolyzed calpain forms. As showed in Figure 2, 2 clear bands, ascribed to μ/m-calpain and its autolyzed form, migrated as a doublet in the casein gel, whereas the μ-calpain band was not detected. These results are consistent with previous studies performed by Kitagaki et al. (2000) and Chang et al. (2016) in which only one calpain isoform was observed in postmortem avian and ostrich muscles. This is likely due to the very limited amount of μ-calpain present in most of the chicken tissues (Lee et al., 2007) and might be partly explained considering that the sensitivity of zymography was previously found not to be high enough to detect all the μ-calpain activity present in postmortem bovine muscles (Hopkins and Geesink, 2009). In addition, since the calcium concentration required for their activation is very low, μ-calpain might be mobilized very soon after slaughter, leading to very low activity 12 h postmortem (Lee et al., 2008). In agreement, μ/m-calpains were identified as the dominant calpain form within the chicken *Pectoralis major* muscles, accounting for the 90% of the total calpain activity (Lee et al., 2007). As shown in Figure 3, the unautolyzed μ/m-calpain activity in the *Pectoralis major* muscles significantly decreased ($P \leq 0.05$) from 100% at 10 h to around 40% at 168 h postmortem, regardless of the occurrence of the WB condition or the intra-fillet sampling position. Concurrently, a 2- to 3-fold increase ($P \leq 0.05$) in the

autolyzed μ/m-calpain activity was measured at longer postmortem times, with the superficial section exhibiting the highest values. Overall, the autolyzed μ/m-calpain activity measured at 10 and 168 h postmortem was 21 and 56%, respectively, of the unautolyzed one measured in NB samples 10 h after slaughtering, which was considered as 100%. Within this context, the absence of significant differences in μ/m-calpain activity might not contribute in explaining the remarkably higher compression forces assessed within the superficial layer of the WB muscles in comparison with their deep and NB (superficial and deep layers) counterparts (Soglia et al., 2017).

Considering its importance in muscle structure integrity and its central role as a target to determine meat tenderness (Koohmaraie and Geesink, 2006), desmin degradation during storage was assessed in order to analyze the influence of the WB condition on the expected degradation of intact desmin taking place within the postmortem period. The relative intensities of intact desmin band and its degradation fragments were quantified, considering as 100% the intensity of the band assigned to intact desmin in NB samples at 10 h postmortem. Only intact desmin (52 kDa) and its 39-kDa-intact rod domain (Baron et al., 2004) were clearly detectable and, thus, quantified (Figure 4). As shown in Figure 5, if compared to NB, a significantly larger ($P \leq 0.05$) amount of desmin was observed within the deep layer of the WB samples at 10 h post-mortem (2.0 fold greater), whereas the superficial portion of the WB muscles exhibited intermediate values. This phenomenon might be partly explained considering that, since according to previous studies (Sihvo et al., 2014; Soglia et al., 2016a), the WB cases exhibited severe histological lesions accompanied by fiber regeneration, a response mechanism involving an increased accumulation of desmin might be hypothesized according to its importance in the structural organization of the sarcomere (Gallanti et al., 1992) and thus also affecting raw meat texture (Mudalal et al., 2015). As the time postmortem proceeded, a sharp decrease of the intact desmin band coupled with a progressive accumulation of its 39-kDa degradation fragment was observed without any significant difference among the groups.

CONCLUSIONS

This study aimed at investigating the effect of a 7-day storage on the proteolytic processes taking place within the NB and WB samples, leading to a deeper knowledge concerning the impact of the WB condition on free calcium concentration, calpain activity, and proteolysis assessed within different intra-fillet sampling locations (superficial vs. deep layer) of the *Pectoralis major* muscles. Overall, the findings of the present study evidenced that, aside from the occurrence of the WB condition and the intra-fillet sampling position, similar electrophoretic profiles, exhibiting an overall increase in the number of detectable bands after aging, were observed. Nevertheless, an evident lack of nebulin was noticed within the superficial layer of the WB cases at 10 h postmortem. Two clear bands ascribed to native μ/m-calpain and their autolyzed forms were detected by casein zymograms. The occurrence of the WB condition and the sampling position did not exert any relevant effect, and native μ/m-calpain activity significantly decreased during storage. Concurrently, an increase in the activity of the autolyzed μ/m-calpain was measured. Interestingly, a significantly higher amount of desmin was observed within both the superficial and the deep layers of the WB cases at 10 h postmortem, although no differences were found in the progressive accumulation of the 39-kDa degradation fragment. In conclusion, the findings of the present study evidenced that the increased hardness that typically affects the WB cases might not be exclusively attributed to the differences in the proteolytic processes taking place within the postmortem period. Thus, further studies are needed in order to investigate and identify the main mechanisms leading to the development of the typical hardened consistency exhibited by the WB samples.

REFERENCES

Ashmore, C. R., P. J. Summers, and Y. B. Lee. 1986. Proteolytic enzyme activities and onset of muscular dystrophy in the chick. Exp. Neurol. 94:585–597.

Baron, C. P., S. Jacobsen, and P. P. Purslow. 2004. Cleavage of desmin by cysteine proteases: Calpains and cathepsin B. Meat Sci. 68:447–456.

Bond, J. J., and R. D. Warner. 2007. Ion distribution and protein proteolysis affect water holding capacity of Longissimus thoracis et lumborum in meat of lamb subjected to antemortem exercise. Meat Sci. 75:406–414.

Chang, Y. S., D. H. Hsu, M. H. Stromer, and R. G. Chou. 2016. Postmortem calpain changes in ostrich skeletal muscle. Meat Sci. 117:117–121.

Chou, R. G. R., T. F. Tseng, K. J. Lin, and J. H. Yang. 1994. Postmortem changes in myofibrillar proteins of breast and leg muscles from broilers, spent hens and Taiwanese country chickens. J. Sci. Food Agric. 65:297–302.

Gallanti, A., A. Prelle, M. Moggio, P. Ciscato, N. Checcarelli, M., Sciacco, A. Comini, and G. Scarlato. 1992. Desmin and vimentin as markers of regeneration in muscle diseases. Acta Neuropathol. 85:88–92.

Hay, J. D., R. W. Curre, F. H. Wolfe, and E. J. Sanders. 1973. Effect of postmortem aging on chicken breast muscle sarcoplasmic reticulum. Food Sci. 38:700–704.

Hillgartner, F. B., A. S. Williams, J. A. Flanders, D. Morin, and R. J. Hausen. 1981. Myofibrillar protein degradation in the chicken. Biochem. J. 196:591–601.

Hopkins, D. L., and G. H. Geesink. 2009. Protein degradation postmortem and tenderization. pp. 149–173 in Applied Muscle Biology and Meat Science, M. Du, and R. J. McCormick, eds. CRC Press, Taylor & Francis Group, Boca Raton, FL.

Huang, J. C., J. Yang, F. Huang, M. Huang, K. J. Chen, X. L. Xu, and G. H. Zhou. 2016. Poultry Sci. 95:2455–2463.

Huff-Lonergan, E., W. Zhang, and S. M. Lonergan. 2010. Biochemistry of postmortem muscle – Lessons on mechanisms of meat tenderization. Meat Sci. 86: 184–195.

Ji, J., and K. Takahashi. 2006. Changes in concentration of sarcoplasmic free calcium during post-mortem ageing of meat. Meat Sci. 73:395–403.

Joo, S. T., R. G. Kauffman, B. C. Kim, and G. B. Park. 1999. The relationship of sarcoplasmic and myofibrillar protein solubility to

colour and water-holding capacity in porcine *longissimus* muscle. Meat Sci. 52:291–297.

Kemp, C. M., and T. Parr. 2012. Advances in apoptotic mediated proteolysis in meat tenderisation. Meat Sci. 92:252–259.

Kitagaki, H., S. Tomioka, T. Yoshizawa, H. Sorimachi, T. C. Saido, S. Ishiura, and K. Suzuki. 2000. Autolysis of calpain large subunit inducing irreversible dissociation of stoichiometric heterodimer of calpain. Biosci. Biotechnol. Biochem. 64:689–695.

Koohmaraie, M., and G. H. Geesink. 2006. Contribution of postmortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. Meat Sci. 74:34–43.

Lee, H. L., V. Sante-Lhoutellier, S. Vigouroux, Y. Briand, and M. Briand. 2008. Role of calpains in postmortem proteolysis in chicken muscle. Poult. Sci. 87:2126–2132.

Lee, H. L., V. Santé-Lhoutellier, S. Vigouroux, Y. Briand, and M. Briand. 2007. Calpain specificity and expression in chicken tissue. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 146:88–93.

Liu, J., M. Ruusunen, E. Puolanne, and P. Ertbjerg. 2014. Effect of pre-rigor temperature incubation on sarcoplasmic protein solubility, calpain activity and meat properties in porcine muscle. LTW-Food Sci. Tech. 55:483–489.

Lowey, S., H. S. Slayter, A. G. Weeds, and H. Baker. 1969. Substructure of the myosin molecule: I. Subfragments of myosin by enzymic degradation. J. Mol. Biol. 42:1–29.

Mudalal, S., M. Lorenzi, F. Soglia, C. Cavani, and M. Petracci. 2015. Implications of white striping and wooden breast abnormalities on quality traits of raw and marinated chicken meat. Animal. 9:728–734.

Nakamura, R. 1973. Estimation of water-extractable Ca in chicken breast muscle by atomic absorption. Anal. Biochem. 53:531–537.

Ouali, A., M. Gagaoua, Y. Boudida, S. Becila, A. Boudjelllal, C. H. Herrera-Mendez, and M. A. Sentrandreu. 2013. Biomarkers of meat tenderness: Present knowledge and perspectives in regards to our current understanding of the mechanisms involved. Meat Sci. 95:854–870.

Pappas, C. T., N. Bhattacharya, J. A. Cooper, and C. C. Gregorio. 2008. Nebulin interacts with CapZ and regulates thin filament architecture within the Z-disc. Mol. Biol. Cell 19:1837–1847.

Paxhia, J., and F. Parrish. 1988. Effect of postmortem storage on titin and nebulin in pork and poultry light and dark muscles. J. Food Sci. 53:1599–1601.

Petracci, M., S. Mudalal, F. Soglia, and C. Cavani. 2015. Meat quality in fast-growing broiler chickens. World Poultry Sci. J. 71:363–374.

Piórkowska, K., J. Nowak, and K. Poltowicz. 2015. The normalisation of *CAPN* gene expression in *M. pectoralis superficialis* in broiler lines differing in growth rate and their relationship to breast muscle tenderness. British Poultry Sci. 56: 452–458.

Pomponio, L., and P. Ertbjerg. 2012. The effect of temperature on the activity of μ- and m-calpain and calpastatin during postmortem storage of porcine *longissimus* muscle. Meat Sci. 91:50–55.

Sakakibara, I., and K. Yagi. 1970. Molecular weight of G-actin obtained by the light-scattering method. BBA-Protein Struct. M. 207:178–183.

Samejima, K., and F. Wolfe. 1976. Degradation of myofibrillar protein components during postmortem aging of chicken muscle. J. Food Sci. 41:250–254.

Schiaffino, S., and C. Reggiani. 1996. Molecular diversity of myofibrillar proteins: Gene regulation and functional significance. Physiol. Rev. 76:371–424.

Sihvo, H. K., K. Immonen, and E. Puolanne. 2014. Myodegeneration with fibrosis and regeneration in the pectoralis major muscle of broilers. Vet. Pathol. 51:619–623.

Soglia, F., J. Gao, M. Mazzoni, E. Puolanne, C. Cavani, M. Patracci, and P. Ertbjerg. 2017. Superficial and deep changes of histology, texture and particle size distribution in broiler wooden breast muscle during refrigerated storage. Poult. Sci. 96:3465–3472.

Soglia, F., L. Laghi, L. Caninico, C. Cavani, and M. Petracci. 2016b. Functional property issues in broiler breast meat related to emerging muscle abnormalities. Food Res. Int. 89: 1071–1076.

Soglia, F., S. Mudalal, E. Babini, M. Di Nunzio, M. Mazzoni, F. Sirri, C. Cavani, and M. Petracci. 2016a. Histology, composition, and quality traits of chicken Pectoralis major muscle affected by wooden breast abnormality. Poult. Sci. 95:651–659.

Sorimachi, H., T. Tsukahara, M. Okada-Ban, H. Sugita, S. Ishiura, and K. Suzuki. 1995. Identification of a third ubiquitous calpain species – Chicken muscle expresses four distinct calpains. Biochim. Biophys. Acta 1261:381–393.

Tatsumi, R., and K. Takahashi. 1992. Calcium-induced fragmentation of skeletal muscle nebulin filaments. J. Biochem. 112:775–779.

Tijare, V. V., F. L. Yang, V. A. Kuttappan, C. Z. Alvarado, C. N. Coon, and C. M. Owens. 2016. Meat quality of broiler breast fillets with white striping and woody breast muscle myopathies. Poult. Sci. 95:2167–2173.

Tomaszewska-Gras, J., F. Schreurs, and J. Kijowski. 2011. Post mortem development of meat quality as related to changes in cytoskeletal proteins of chicken muscles. Br. Poult. Sci. 52:189–201.

Turner, P., J. T. Westwood, C. M. Regan, and R. A. Steinhardt. 1988. Increased protein degradation results from elevated free calcium levels found in muscle from mdx mice. Nature 335: 735–738.

Zambonelli, P., M. Zappaterra, F. Soglia, M. Petracci, F. Sirri, C. Cavani, and R. Davoli. 2016. Detection of differentially expressed genes in broiler Pectoralis major muscle affected by white striping – Wooden breast myopathies. Poult. Sci. 95:2171–2785.

Zhao, L., N. Jiang, M. Li, M. Huang, and G. Zhou. 2016. Partial autolysis of μ/m-calpain during post mortem aging of chicken muscle. Animal Sci. J. 87:1528–1535